Assessment of Human Lung Macrophages After Exposure to Multi-Walled Carbon Nanotubes Part II. DNA Damage

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Due to the widespread production and use of carbon nanotubes in almost every area of science (i.e., drug delivery, biosensors, fuel cells and thermal management systems), they are receiving considerable attention for their novel mechanical, electrical and chemical properties. At this time of high exposure potential, it is critical to ascertain the biological impact of these materials on likely target organs, tissues and cells, such as those of the lung. The aim of this study was to evaluate the degree of DNA damage to human lung macrophage (U937) cells after exposure to unpurified or acid-purified multi-walled carbon nanotubes. Cells were incubated with multi-walled carbon nanotubes and assessed for DNA damage response via fluorescent staining and a virtual gel electrophoresis technique. The results demonstrate that multi-walled carbon nanotubes may induce an early (2–4 h) stress response and contribute to DNA mismatch during cell replication. Similarly, after 24 h, the direct assessment of DNA damage revealed an overall reduction and degradation in total cellular DNA. Therefore, before nanomaterials are fully accepted and integrated into biological systems, they will continue to undergo further scrutiny at various stages of their processing (i.e., before and after purification) and with models ranging from simple to complex (i.e., cells vs. whole animals) to gain a better understanding between their physicochemical properties and bio-effects.

Keywords: U937 Cell, MWNTs, MWNTs-COOH, DNA Damage.

1. INTRODUCTION

Since the discovery of carbon nanotubes (CNTs) about two decades ago,[1] there has been intensive research activity in both fundamental and applied frontiers. Carbon nanotubes are being incorporated into almost every area of science for applications that require their extraordinary mechanical, electrical, chemical, and more recently investigated, biological properties.[2] Applications developed from CNTs include drug delivery devices, biosensors, fuel cells, dry adhesives, and thermal management systems.[3–8]

Because inhalation is a common route of airborne nanomaterial exposure, most studies have focused on organs such as the lungs, which due to their large surface area are common targets of many toxicants.[9–14] Other studies of lung cells typically include epithelial cells which line the lungs or macrophages, which are responsible for removing foreign debris and inflammatory responses.[15–18] Previous studies in our laboratory[19] and others[17] have shown that nanoparticles can alter the phagocytic response of macrophages, which may have implications in disease conditions. In the case of CNTs, lesions resulting from the persistent interaction between the cell and trapped materials in the interstitium of the lung, induction of ROS generation, increases in cell death, enhanced DNA damage as well as local and systemic responses such as cardiovascular, neurologic, and immunologic toxicity have been demonstrated.[10, 11, 20–23] The production of ROS by macrophages is one mechanism to kill foreign entities such as pathogens and several studies suggest that ROS can regulate the production of cytokines in macrophages through mechanisms that are dependent on NF-kB.[32, 33] Free radicals and ROS also can chemically alter DNA bases and cause DNA damage within cells. For example, our previous

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research demonstrated a cellular DNA damage response to MWNTs in mouse embryonic stem (ES) cells, which was indicated by two key double strand break repair proteins, Rad 51 and XRCC4 as well as an increase in p-53 expression level.[28]

In contrast, there are many studies utilizing CNTs in biocompatible ways for medical purposes.[24] Therefore, further investigation into the factors that influence nanomaterial genotoxicity, such as purification procedures and resultant surface chemistry continue to be elucidated.[25] Aside from surface chemistry, differences including cell type (e.g., macrophage vs. keratinocyte or fibroblast); CNT properties (synthesis method, # walls, purity, dimensions, etc.); solutions for dilution and dispersion; dosing concentrations, exposure times; and methods for assessing toxicity (e.g., MTT assay, NR assay, TNF-α production) may be responsible for the different results obtained thus far for CNT biocompatibility.[26, 27]

2. RESULTS AND DISCUSSION

In the current study, we chose the human lung macrophage cell line (U937) as a model to investigate the potential of unpurified multi-walled carbon nanotubes (MWNTs) and acid purified multi-walled carbon nanotubes (MWNT-COOH) to produce DNA damage. The unpurified MWNTs

and acid-purified MWNT-COOH were characterized and assessed for cytotoxicity in Part I. The DNA damage response after exposure to low concentrations (5 μg/ml) of MWNTs versus MWNT-COOH was examined at early time points (2–4 h) and p-53 activity level was analyzed by immunofluorescent staining. The p53 tumor suppressor protein plays a major role in cellular response to DNA damage and other genomic aberrations. Activation of p53 is known to be modulated by protein phosphorylation, which transforms the p53 protein from a latent to an active conformation. Activation of p53 can lead to either cell cycle arrest and DNA repair or apoptosis. As can be seen in Figure 1, the cells were stained green for phosphorylated p53 protein and stained red for cluster of differentiation 68 (CD68) as a cell marker, which is a glycoprotein which binds to low density lipoprotein expressed on monocytes/macrophages. The untreated control (Fig. 1(A)) shows the CD68 marker and low p53 protein levels. In contrast, cells exposed to MWNT (Fig. 1(B)) or MWNT-COOH (Fig. 1(C)) show increased p53 staining within 2 hours at the low concentration of 5 μg/ml.

To further investigate the potential of MWNTs and MWNT-COOH to induce specific kinds of DNA damage, the expression of the key mismatch repair protein MSH2 was examined. The MSH2 gene provides instructions for making a protein that fixes mistakes that are made when
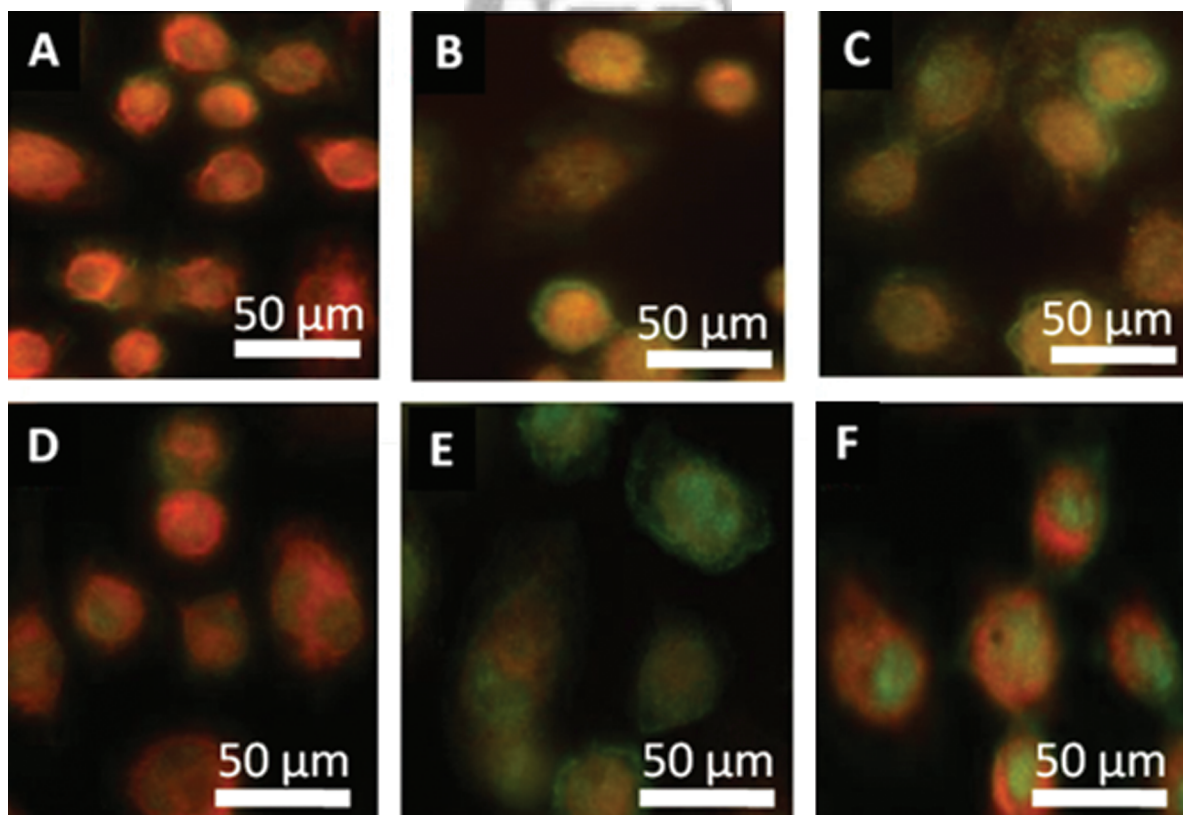

Fig. 1. Immunofluorescent Staining. U937 cell stained red with CD68 antibody and green for p-53 antibody (A–C, 2 h) or MSH2 antibody (D–F, 2 h). (A, D) Control, (B, E) MWNTs 5 μg/ml, (C,F) MWNT-COOH 5 μg/ml.

DNA is copied (DNA replication) in preparation for cell division. As can be seen in Figures 1(D–F), the cells were stained green for MSH2 protein and stained red for CD68 as a cell marker. The untreated control (Fig. 1(D)) shows the CD68 marker and low MSH2 protein levels. In contrast, cells exposed to 5 μg/ml MWNTs displayed increased MSH2 protein levels within 2 h (Fig. 1(E)) and greater MSH2 protein staining than cells exposed to MWNT-COOH under the same conditions (Fig. 1(F)). Therefore, although the p53 staining was similar for both MWNTs and MWNT-COOH after 2 h, the greater MSH2 staining for the MWNTs after 2 h suggests that the unpurified MWNTs have the potential to induce greater damage (and concurrent ROS generation) compared to the acid-purified MWNT-COOH after cumulative incubation for 2 h.

In support of the fluorescent antibody images, quantification of the p-53 and MSH2 protein levels were performed. There was a slight increase in p53 protein level compared to the control for the MWNTs and MWNT-COOH-treated cells after 2 h (Fig. 2). However, after 4 h,

the cells exposed to MWNTs had elevated p53 protein levels at both the low and high concentrations (5, 50 μg/ml) compared to MWNT-COOH further confirming the accumulative damage potential of unpurified MWNTs versus MWNT-COOH. These observations suggest that unpurified MWNTs could potentially lead to more DNA damage more than acid-purified MWNTs-COOH over time, as evidenced by the induction and accumulation of the p53 tumor suppressor protein after 4 h. Similarly, when the levels of MSH2 protein were quantified after 2 h, (Fig. 2(B)), there was an increase in MSH2 levels after the cells were exposed to the low dose (5 μg/ml) MWNTs, but not MWNT-COOH. These low dose effects are important because they may signify differences in nanomaterial physiochemical properties (i.e., aggregation state, impurity content, etc.), which can skew nanotoxicity dose-effects. In this case, at the high concentration of MWNTs, the p53 and MSH2 levels did not respond at the same dose-effects of nanotoxicity suggesting that the agglomeration of MWNTs at the higher concentration may lead to reduced cell uptake and down-stream bio-effects.

To confirm the direct damage of cellular DNA by MWNTs and MWNT-COOH after a later time point of 24 h, a novel virtual gel electrophoresis technique was performed. The results suggest that after 24 hours of incubation with 5 μg/ml concentrations of MWNTs and MWNT-COOH that the overall total DNA amounts are reduced and degraded compared to untreated cells (data not shown).

3. SUMMARY AND CONCLUSIONS

We have previously shown (Part I.) that MWNTs and MWNT-COOH can accumulate in human lung macrophage cells to different degrees without producing overt cell toxicity under these conditions (5–50 μg/ml MWNT or MWNT-COOH, 2–24 h). Furthermore, there were morphological alterations at low doses of MWNT-COOH and significant ROS production for MWNT at high doses indicating a potential, although different, cellular stress response for both materials. In Part II. of this study we found a cellular stress response (p53) and DNA mismatch (MSH2) potential as early (2–4 h) mechanistic responses to low doses of unpurified MWNTs. There was also direct evidence for DNA damage by virtual gel electrophoresis after 24 h of incubation with either MWNTs and MWNT-COOH (data not shown). In support of this data, recent evidence suggests that p53 can also induce the expression of proteins that function to lower ROS levels and that this antioxidant function of p53 is important in preventing DNA damage and tumor development under low-stress conditions[34] (Sablina et al., 2005). Therefore, the ROS demonstrated by MWNTs in Part I. may be linked to p53 expression and DNA damage. However, before nanomaterials are fully accepted and integrated into
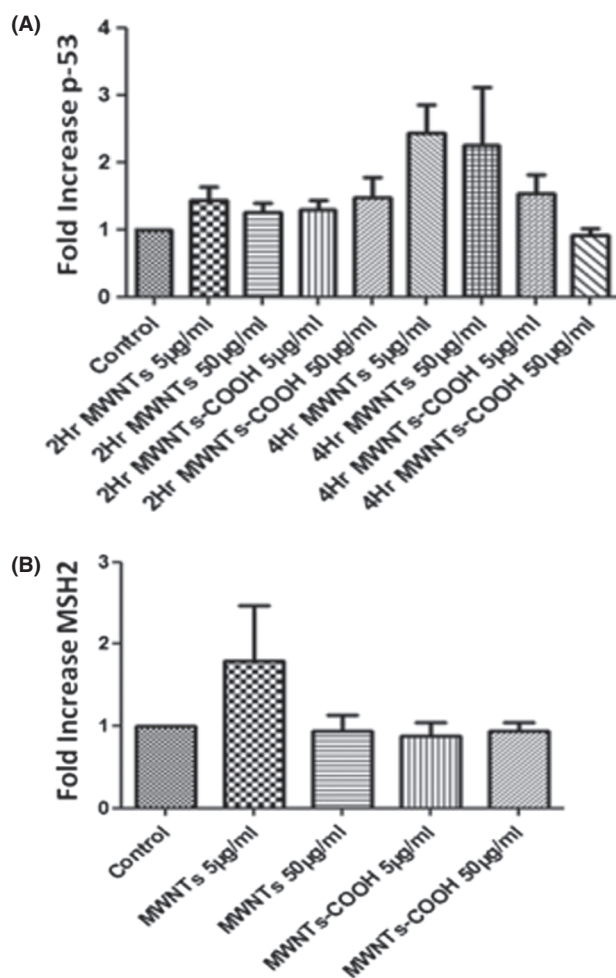

Fig. 2. Quantification of (A) p-53 (2 h and 4 h) or (B) MSH2 levels (2 h) after exposure to MWNTs or MWNT-COOH.

Table I. Summary of results.

Observation/ Assay	Description	MWNTs	MWNT-COOH
p-53	Stress response protein, 2–4 h	Increase after 4 h for 5 and 50 μg/ml	No chage
MSH$_2$	DNA base mismatch repair protein, 2 hvs3	Increase at low 5 μg/ml concentration	No chage
Virtual gel electrophoresis	Direct DNA damage, 24 h	Overall total DNA amounts are reduced and degraded	Overall total DNA amounts are reduced and degraded

biological systems, they will continue to undergo further scrutiny at various stages of their processing (i.e., before and after purification) and with models ranging from simple to complex (i.e., cells vs. whole animals) to gain a better understanding between their physicochemical properties and bio-effects.

4. MATERIAL AND METHODS

4.1. Cell Culture

Human alveolar macrophage cells (U937, ATCC) were culture in T-75 flasks and incubated with 5% CO$_2$ at 37 °C. The U937 cells are a monocyte cell line that can be stimulated to mature into macrophages, which play the major role in both non-specific and specific defense mechanisms in the body. The U937 cells were maintained in RPMI media supplemented with 10% HI FBS (Invitrogen) and 1% penicillin and streptomycin (Invitrogen). The cells were stimulated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA) for 48 hours to allow differentiation into human alveolar macrophage cells before dosing.

4.2. MWNT and MWNT-COOH Dosing Protocol

MWNTs were purchased from Tsinghua and Nanfeng Chemical Group Cooperation, China. MWNT-COOH was obtained and characterized with the same procedure as in Part I. Briefly, MWNT stock solutions were prepared at concentrations of 1 mg/ml in water, then briefly sonicated for 2–3 minutes with intermittent rest for ~3 minutes and repeated 3–4 times.

4.3. Immunofluorescent Staining and Quantification

Macrophages grown on two-chambered slides and incubated with MWNTs and MWNTs-COOH were fixed with 4% paraformaldehyde (EMS) for 10 minutes, permeabilized

in PBS containing 0.2% Triton for 5 minutes, and blocked with 1% BSA (Invitrogen) for 1 h. The slide was stained with U937 cell marker CD68 (Santa Cruz) and anti cell cycle checkpoint protein p-53 (Santa Cruz) and an antibody for DNA repair protein MSH2 (Santa Cruz) at 4 °C overnight. After washing with 1× PBS, the cells were stained with fluorescent-labeled secondary antibody Alexa 488 and 548 (1:1000, Abcam) at room temperature for 45 minutes. The coverslips were mounted with Prolong Antifade reagent (Invitrogen) overnight to cure. Images were acquired on a confocal microscope (BD pathway 435) using a 40× oil lens (Olympus 1 × 71). For immunofluorescent quantification, the procedure was the same as above with the exception of using black 96-well imaging plates instead of two-chambered slides. The data are represented as the average of triplicate + the standard deviation.

4.4. Caliper GXII Virtual Gel Electrophoresis for DNA Damage Assessment

After 24 hours of incubation with 5 μg/ml concentrations of MWNTs and MWNT-COOH, cells were harvested by 2% trypsin digestion and centrifuged at 5000x rpm for 5 minutes. DNA was extracted from the cell pellets and run on the Caliper GXII Labchip machine (Caliper Life-Sciences) according to the manufacturer's protocol. Virtual gel images were examined for DNA degradation represented by band fragmentation.

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